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Polarity

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13. ABSTRACT (Maximum 200 Words) ErbB2 is overexpressed in approximately 25% of breast cancers. ErbB2 homodimers can cause an increase in cell proliferation and a disruption of cell polarity, both characteristics of early cancer stages. In order to dissect the role of ErbB2 biochemistry on cell polarity we have constructed two series of mutations in the autophosphorylation sites of chimeric Neu (rat ErbB2). The chimeric receptor is used in order to create an inducible system of Neu homodimers. In one series 4 of the 5 autophosphorylated tyrosines are active. In the other only one of these tyrosines is active (Y mutants). Preliminary results suggest that different Y mutations can cause distinct cell polarity disruption phenotypes. More in depth studies of these phenotypes are planned for the near future, including some preliminary studies to look at possible changes in the three major polarity complexes - Par, Crumbs and Lgl. In order to investigate the role localization plays in ErbB2's ability to promote proliferation and disrupt polarity, we have also constructed a series of mutations that cause mislocalization of chimeric ErbB2. Preliminary data suggests that ErbB2 cannot phosphorylate from the apical surface, but must be located on the lateral surface-where wild type ErbB2 normally localizes - or in a non-polarized cell. After more in depth testing of this hypothesis, we shall attempt to find the factor(s) responsible for the inability of ErbB2 to phosphorylate from the apical surface.				
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ErbB2 affects on Cell Polarity

Introduction

The ErbB receptor tyrosine kinase family has long been implicated in cancer. Of its four members – ErbB1, ErbB2, ErbB3, and ErbB4 – the first three are frequently found overexpressed in breast cancer. In particular overexpression of ErbB2 in vivo is correlated with poor clinical prognosis [1, 2]. Although ErbB2 does not form homodimers at normal concentrations, it will form homodimers when highly overexpressed. In experimental models both overexpression of ErbB2 and inducible ErbB2 homodimers have been shown to disrupt cell polarity and to reinitiate proliferation[3], phenotypes characteristic of early stage cancers.

Our lab uses a unique ErbB chimeric receptor, in which both the extracellular and transmembrane domains have been replaced with corresponding domains from the p75 low affinity NGFR receptor. Dimerization is mediated by an intracellular FKBP domain which recognizes the synthetic ligand AP1510. This allows for select activation of a specific ErbB family member, without activation of any of the other ErbB family members, thereby allowing for study of a downstream affects from a single ErbB receptor [4].

This grant proposed, in part, to determine the ability of ErbB2 to regulate the Par complex, one of the three known complexes important in establishing cell polarity. Data obtained in our lab suggests that ErbB2 does, indeed, regulate the Par complex. In order to dissect the ability of ErbB2 to regulate this complex we will look both at the roles played by biochemistry regulated by individual autophosphorylated tyrosines and at the role of ErbB2 localization in the plasma membrane.

There are 5 tyrosines in ErbB2's carboxy terminal tail that are autophosphorylated upon ErbB2 dimerization. As a receptor tyrosine kinase, ErbB2 must use these phosphorylated tyrosines to interact with downstream effectors. Indeed, other labs have observed that autophosphorylation site (Y) mutants YB, YC, YD and YE all promote proliferation in fibroblasts [5] and that each tyrosine binds to a different effector [6]. In this project we shall use two series of mutations of the autophosphorylated tyrosines to determine (1) if the disruption of polarity caused by ErbB2 is dependent on one or more individual tyrosines, (2) which of these tyrosines regulate the Par complex, (3) if the other two major polarity complexes – Crumbs and Lgl – are also regulated by ErbB2 and (4) if it is possible to obtain the wild type ErbB2 disruption of polarity phenotype by manipulating the polarity complexes themselves.

In addition to investigating the role of individual tyrosines in the regulation of polarity it will be important to understand the reciprocal relationship – does polarity or lack thereof affect ErbB2's ability to function? ErbB2 normally localizes to the lateral membrane where, at high concentrations, it can homodimerize, resulting in its phosphorylation and downstream signaling. This signaling results in disruption of cell polarity and promotion of proliferation. Previous work has shown that mutations in 8 or 12 amino acids in the intracellular juxtamembrane region of ErbB2 can cause lateral/apical and apical localization, respectively, of the receptor [7]. These mislocalization mutants can be used to examine the regulatory affect, if any, that established epithelial polarity has on the ability of ErbB2 to promote cell proliferation and disrupt cell polarity.

Body

Using the chimeric receptor as "wild type" ErbB2 we have created three distinct series of mutations in order to investigate the role of individual autophosphorylated tyrosines and ErbB2 localization in its ability to disrupt cell polarity.

Autophosphorylated Tyrosine Mutants

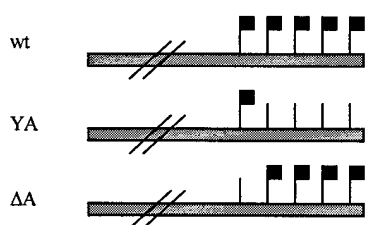


Figure 1: Examples of Y and Δ mutants. In Y mutants all but one of the autophosphorylation sites is mutated to phenylalanine, whereas in delta mutants 4 autophosphorylation sites remain intact and only one is mutated to phenylalanine.

Autophosphorylation site mutants will be used to dissect the role of ErbB2 biochemistry in the disruption of cell polarity. To date we have created two series of mutations of these tyrosine sites in the rat homolog of ErbB2, Neu. The high homology of ErbB2 and Neu, along with their similar phenotypes indicates that these homologs have highly conserved functions. The first series of mutations in Neu, called deltas, has one of the five sites mutated to phenylalanine. The second series, referred to as the Ys, has been mutated so that 4 of the 5 tyrosine sites are inactive phenylalanines, leaving only one tyrosine active (Figure 1). In addition control mutations have been created in which all tyrosines are mutated to phenylalanine or the kinase activity has been knocked out. All these mutations and a wild type form of Neu have been used to create pools of Mandin Darby

Canine Kidney (MDCK) cells expressing the various constructs. Preliminary data indicates that polarity phenotypes observed for Y mutants can be separated into two groups (Figure 2), each one distinct from the phenotype seen with activation of wild type chimeric Neu. Once these phenotypes are better characterized it will be important to discover how the tyrosines differentially regulate polarity, what combination of polarity regulation is necessary to obtain the wild type polarity phenotype and further to investigate the relationship between the loss of polarity and the reinitiation of proliferation.

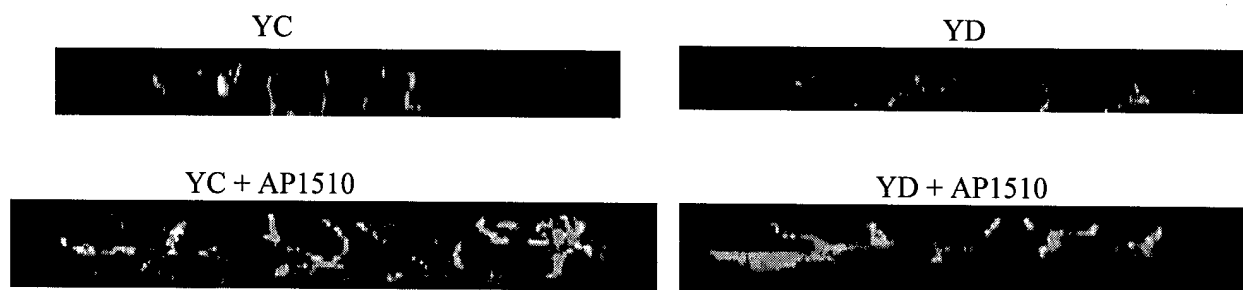


Figure 2: Different polarity phenotypes of autophosphorylation site mutants. Here are two examples of the different affects a single tyrosine can have on the cell polarity marker protein Zo-1 (red). YC when activated causes two layers of cells to form, each with proper Zo-1 staining and with a layer of ErbB2 HA+ (green) membrane between them. On the other hand, YD shows multilayering upon stimulation, but only the top layer of cells expresses Zo-1. These are very preliminary results.

Juxtamembrane Mutations

In epithelial cells, proteins are separated into various areas, resulting in what is termed cell polarity. ErbB2 is one of these proteins that is not ubiquitously present, but is instead localized to the lateral membrane. Indications from our lab suggest that ErbB2 regulates the Par complex, which is also laterally localized. This prompts the question - Is this proximity between ErbB2 and its effectors necessary for proper signaling? In order to address this question we have created a human form of the chimeric ErbB2 molecule expressing juxtamembrane mutations that cause ErbB2 mislocalization.

The original mutations of the juxtamembrane region were very generously provided by Clive Dickson as full length constructs of human ErbB2. A simple exchange of the intracellular domains of the chimeric receptor and the full length human ErbB2 constructs has been used to create human wild type and mutant forms of chimeric ErbB2. Immunofluorescence was used to confirm the proper localization of each chimeric ErbB2. In addition, the inducible activity of the receptors was determined by comparing the phosphorylation of receptor with and without stimulation (Figure 3). No previous analysis of the kinase activity of juxtamembrane mutants has been reported, leaving two options - the mutations introduced to these receptors had negatively affected their kinase ability or lateral localization is necessary for the autophosphorylation of ErbB2.

In order to simultaneously test these hypotheses the receptors were stimulated in cells in suspension, which have lost their polarity and so will have similar localization for both wild type and mutant ErbB2 chimeras. A significant increase in the inducible phosphorylation was seen, suggesting that differences between the apical and lateral membranes regulate ErbB2's ability to autophosphorylate. In order to confirm this future studies will need to be done with these receptors in a variety of cell lines, both polarized and unpolarized. In the meantime an in vitro kinase assay is underway to confirm that the mutant receptors are not deficient in kinase activity and that any variation seen in non-polarized cells is due to a residual biological factor and not to an intrinsic decrease in kinase activity.

If the preliminary indications that ErbB2 must be localized laterally in order to properly autophosphorylate are confirmed, additional experiments will be done to identify the factors that are responsible for facilitating ErbB2 autophosphorylation. These experiments will include co-immunoprecipitation of ErbB2 and use mass spectroscopy to characterize binding partner that may facilitate ErbB2 autophosphorylation.

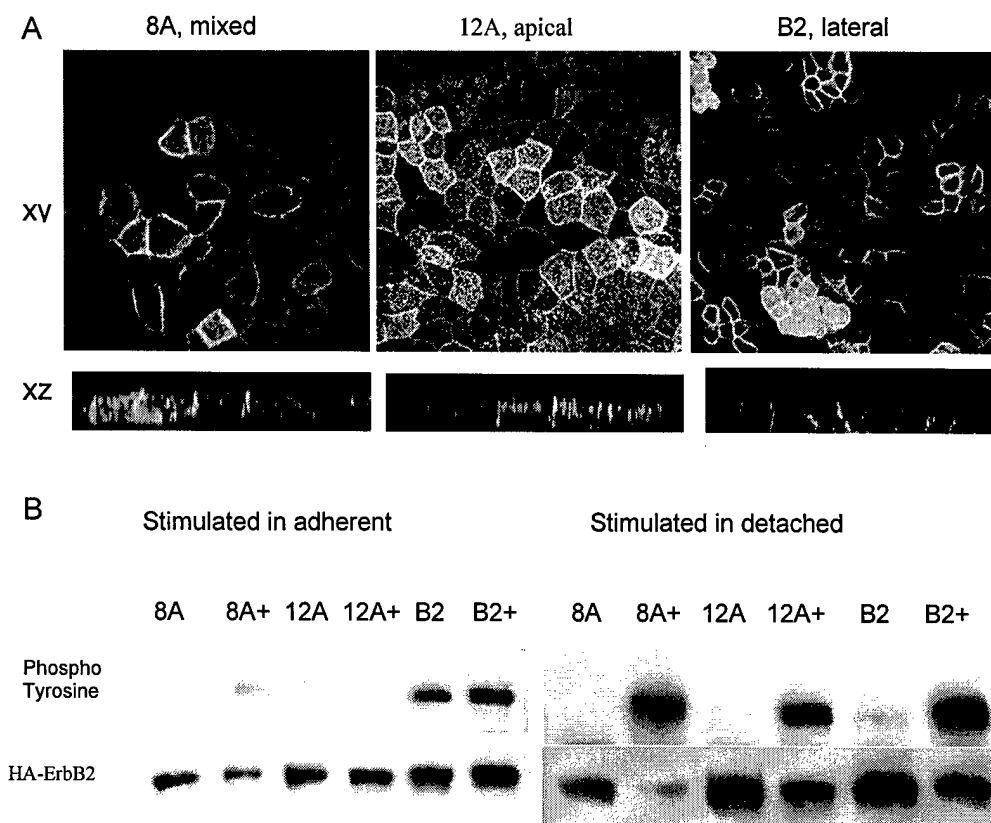


Figure 3. Juxtamembrane Mutant ErbB2 receptors localizes as expected, but do not autophosphorylate well in adherent cells. MDCK cells expressing chimeric forms of ErbB2 that localizes to the lateral (B2), lateral/apical (8A) or apical (12A) surfaces. (A) The xy view shows the top down view of the cells whereas the xz view shows a cross section. ZO-1, a tight junction associated molecule, is stained red. The HA tag of the chimeric receptor is stained green. The nuclei are labeled blue with DAPI. (B) Activity of the receptors as shown by the ability to autophosphorylate on tyrosine. The left panel shows the ability of receptors in adherent, and therefore polarized, MDCKs. The right panel shows the ability of receptors in detached, and therefore not fully polarized, MDCKs. HA is used to show the amount of receptor present in each lane.

Key Accomplishments

- creation of a series of mutations in chimeric Neu where individual autophosphorylate tyrosines are mutated to phenylalanine, leaving the four other autophosphorylated tyrosines intact (delta A (dA), dB, dC, dD, dE).
- creation of a series of mutations in chimeric Neu where 4 of the 5 autophosphorylated tyrosines are mutated to phenylalanine (YA, YB, YC, YD, YE)
- creation of chimeric Neu which lacks all tyrosines that can be autophosphorylated (Y0)
- creation of a kinase dead chimeric Neu (kn)
- creation of chimeric ErbB2, wild type (B2) and mislocalization mutants (8A and 12A)
- development of epithelial cell lines expressing each of these mutations using retroviral infection
- initial biochemical analysis to determine that all delta, Y and juxtamembrane mutants can be induced to autophosphorylate
- FACS sorting of cells expressing YB, YC, YD, YE, B2, 8A and 12A in order to obtain populations with homogenous expression levels
- Preliminary results that suggest that although none of the Y mutants alone can recreate the wild type polarity disruption, YB, YC, YD, and YE each seem to disrupt polarity to some degree with perhaps different roles in polarity disruption
- Preliminary results that suggest the possibility that ErbB2 lateral localization may be necessary for its proper autophosphorylation

Conclusion

This past year has been used to create a wide range of mutations in ErbB2 or its rat homolog Neu that have been put into epithelial cells and are now ready to be analyzed. Preliminary results indicate that a single tyrosine is not responsible for the full effect Neu has on cell polarity since none of the individual tyrosines can recreate that phenotype. More over, the differences seen in some cases between individual tyrosines seems to suggest that they may play distinct roles in the regulation of cell polarity. Now that all tools are ready, experiments to dissect the role of ErbB2 biochemistry on cell polarity can proceed.

Preliminary results with the juxtamembrane mutants indicate that location of ErbB2 within a polarized cell may actually regulate its ability to autophosphorylate. This intriguing result still needs to be tested in a variety of other cell lines in order to confirm this result. However, if confirmed it will become important to determine what factor(s) are responsible for this regulation.

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